Exhibit O

User Manual

Make Your Own "Mate & Plate™" Library System User Manual

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Table of Contents

I. Introduction & Protocol Overview 4
II. List of Components 5
III. Additional Materials Required 7
IV. List of Abbreviations 8
V. Host Strain Information 9
VI. Control Experiments 10
A. General Considerations 10
B. Protocol: Control for Homologous Recombination-Mediated Cloning Protocol 10
VII. Generating the cDNA for Your Library 11
A. Protocol: First Strand cDNA Synthesis 11
B. Protocol: Amplify cDNA Using Long Distance PCR (LD-PCR) 13
C. Protocol: Purify ds cDNA with CHROMA SPIN™ TE-400 Columns 14
VIII. Construction of a Two-Hybrid Library 16
A. General Considerations for Two-Hybrid Library Construction & Screening 16
B. Protocol: Creating a Mate & Plate Library 16
IX. References 18
X. Troubleshooting Guide 19
Appendix A: Library Titering 21
Appendix B: Plasmid Information 22
Appendix C: Yeast Growth Media & Supplements 23
Appendix D: SMART™ Technology Overview 25

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Table of Contents continued

List of Figures

Figure 1. Use yeast biology to construct a Mate & Plate library. 4
Figure 2. Mate & Plate Library construction overview. 11
Figure 3. High-quality cDNA generated using SMART cDNA synthesis. 14
Figure 4. CHROMA SPIN column and collection tubes. 15
Figure 5. Restriction Map and Cloning Sites of pGADT7-Rec. 22

List of Tables

Table I: Yeast Host Strain Genotypes 9
Table II: Phenotype Testing on Various SD Media 9
Table III: Relationship between Amount of RNA and Optimal Number of Thermal Cycles 13
Table IV: Components of Yeast Media Set 2 & Yeast Media Set 2 Plus 23
Table V: Individual Yeast Media Pouches for Matchmaker Gold Protocols 23
Table VI: Additional Media & Media Supplements Required for a Two-Hybrid Screen 24

I. Introduction & Protocol Overview

Yeast two-hybrid systems are primarily used for screening a complete library of proteins (prey) for interaction with a specific protein of interest (bait). Since traditional library manufacture is time-consuming and labor-intensive, Clontech has developed the **Mate & Plate™ Libraries,** a series of ready-to-go libraries that require simple co-culturing of a library strain with your bait strain followed by selection on appropriate minimal medium.

Our **Make Your Own "Mate & Plate™" Library System** (Cat. No. 630490) enables you to "make your own Mate & Plate Library" just the way we do it using a simple and highly efficient protocol.

Library construction takes place directly in our library yeast strain Y187, utilizing the highly potent homologous recombination machinery of *Saccharomyces cerevisiae* (Figure 1). There is no need for labor-intensive library cloning, amplification, and harvesting in *E.coli*. This system uses SMART™ cDNA synthesis technology, which allows you to construct cDNA libaries from any tissue source starting with as little as 100 ng of total RNA (see Appendix D for an overview of SMART technology).

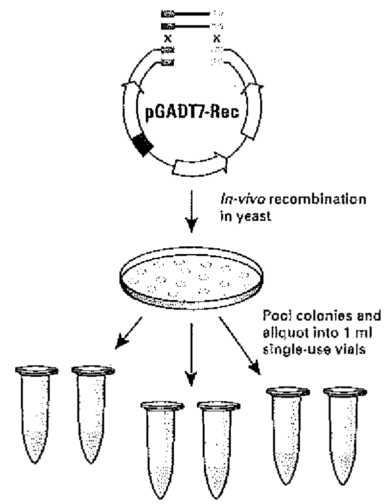

Figure 1. Use yeast biology to construct a Mate & Plate library. Mate & Plate libraries are made directly in yeast, via *in vivo* recombination between your cDNA and the Matchmaker prey vector pGADT7-Rec. First, use SMART cDNA synthesis technology to create a cDNA pool with end sequences homologous to the prey vector pGADT7-Rec. Then transform Y187 Yeast Strain and allow the yeast to perform a recombination step between the linear prey vector and the cDNA. Colonies are pooled, mixed, and aliquoted into multiple vials. Each single 1 ml vial can be used for a two-hybrid screen.

About this Manual

This manual describes how to make your own library and divide it into aliquots in preparation for a yeast two-hybrid screen. For detailed procedures regarding the library screening process itself, please see our Matchmaker Gold Yeast Two-Hybrid System User Manual (PT4084-1) at **www.clontech.com** The **Matchmaker Gold Yeast Two-Hybrid System** is sold separately as Cat. No. 630489.

Matchmaker Library Construction Overview

The library construction process consists of the following steps (see Figure 1):

- **Step 1.** Generate cDNA from RNA using SMART™ technology
- **Step 2.** Cotransform cDNA and linearized pGADT7-Rec vector into Yeast Strain Y187, and plate on SD/–Leu Agar medium
- **Step 3.** Wait 4–5 days, harvest yeast, and pool all colonies in rich broth medium.
- **Step 4.** Divide into aliquots and freeze.
- **Step 5.** Use a single 1 ml aliquot for each library screen [see the Matchmaker Gold Yeast Two-Hybrid System User Manual (PT4084-1)].

II. List of Components

Make Your Own "Mate & Plate" Library System (Cat. No. 630490)

This kit contains sufficient reagents (listed below) to make five two-hybrid libraries.

Store Yeast Media Pouches, deionized H_2O, CHROMA SPIN™ Columns, NaCl Solution, NaOAc, LiAc, PEG, TE Buffer, and YPD Plus Medium at room temperature.

Store yeast strain, Control Poly A+ RNA, and the SMART III Oligo at –70°C.

Store all other reagents at –20°C.

First-Strand cDNA Synthesis

- 10 µl SMART III Oligo
  (10 µM; 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3')

NOTE: The SMART III Oligo is a modified oligo.

- 10 µl CDS III Primer
  (10 µM; 5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)$_{30}$VN-3')*
- 10 µl CDS III/6 Primer
  (10 µM; 5'-ATTCTAGAGGCCGAGGCGGCCGACATG-NNNNNN-3')

NOTE: N = A, G, C, or T; V = A, G, or C

- 10 µl SMART MMLV Reverse Transcriptase (200 U/µl)
- 7 µl RNaseH
- 300 µl 5X First-Strand Buffer
- 165 µl DTT (100 mM)
- 5 µl Control Poly A+ RNA (Mouse Liver; 1 µg/µl)
- 50 µl dNTP Mix (10 mM each)

cDNA Amplification

- 50 µl 5' PCR Primer
  (10 µM; 5'-TTCCACCCAAGCAGTGGTATCAACGCAGAGTGG-3')
- 50 µl 3' PCR Primer
  (10 µM; 5'-GTATCGATGCCCACCCTCTAGAGGCCGAGGCGGCCGACA-3')
- 500 µl Melting Solution

II. List of Components continued

cDNA Purification

- 10 CHROMA SPIN+TE-400 Columns
- 300 µl Sodium Acetate (3 M)

Two-Hybrid Library Construction

- 25 µg pGADT7-Rec Cloning Vector (SmaI-linearized; 500 ng/µl)
- 20 µl SV40 Large-T PCR fragment (25 ng/µl)
- 0.5 ml Y187 Yeast Strain
- 50 ml NaCl Solution (0.9%)

- Make Your Own "Mate & Plate" Library System User Manual (PT4085-1)
- pGADT7-Rec Vector Information (PT3530-5)

Complimentary Yeast Media Pouches

- 1 x 0.5 L YPDA Broth
- 1 x 0.5 L YPDA with Agar
- 1 x 0.5 L SD/–Leu with Agar

Yeastmaker™ Yeast Transformation System 2 (also available separately as Cat No. 630439)

- 50 ml 1 M LiAc (10X)
- 50 ml 10X TE Buffer
- 50 ml YPD Plus Liquid Medium
- 20 µl pGBT9 (0.1 µg/µl; positive control plasmid)
- 2 x 1 ml Yeastmaker Carrier DNA, denatured (10 mg/ml)
- 2 x 50 ml 50% PEG 3350

- Yeastmaker Yeast Transformation System 2 User Manual (PT1172-1)

III. Additional Materials Required

Advantage® 2 Polymerase Mix (100 rxns) (Cat. No. 639201)

Accessory Kits

- **Yeastmaker Yeast Transformation System 2** (supplied with your system—see Section II; also sold separately as Cat. No. 630439)
- **Easy Yeast Plasmid Isolation Kit** (50 preps; Cat. No. 630467)

Tools for Plating Yeast

- Tools for plating yeast include a sterile glass rod—and a bent Pasteur pipette or 5 mm glass beads for spreading cells on plates. (Use 5–7 beads per 100 mm plate, or 15–20 beads for a 150 cm plate).

Miscellaneous Reagents & Media

- 3 M sodium acetate, pH 5.3
- ice-cold ethanol (95–100%)
- YPDA/25% Glycerol (Freezing Medium; see Appendix C, Section C)

Yeast Media Pouches Required for Library Construction (also see Appendix C)

- YPDA Broth (10 x 0.5L) Cat. No. 630306
- YPDA with Agar (10 x 0.5L) Cat. No. 630307
- SD/-Leu with Agar (10 x 0.5L) Cat. No. 630311

Additional Yeast Media for Two-Hybrid Screening
[also see the Matchmaker Gold Yeast Two-Hybrid System User Manual (PT4084-1)]

Table IV (in Appendix C) lists the components of the **Yeast Media Set 2** (Cat. No. 630494) and the **Yeast Media Set 2 Plus** (Cat. No. 630495). These media sets contain a complete assortment of mixes for preparing eight specialized broth and agar media, designed for use with the **Matchmaker™ Gold Yeast Two-Hybrid System**, in convenient, "ready-mixed" foil pouches. The **Yeast Media Set 2 Plus** also contains the additional media supplements **Aureobasidin A** and **X-α-Gal**, which are required for a two-hybrid screen. Table V (in Appendix C) contains information for purchasing each of the media mixes separately, in packs of 10 pouches, and Table VI (in Appendix C) contains preparation instructions for all additional required media supplements and information for purchasing Aureobasidin A and X-α-Gal separately.

Additionally, the following should be considered when culturing yeast for a two-hybrid screen:

- **SD medium** (synthetically defined medium) is minimal media that is routinely used for culturing *S. cerevisiae*. **SD base** supplies everything that a yeast cell needs to survive (including carbon and nitrogen sources). Essential amino acids, which are added to SD base to create minimal medium, are already included premixed in Clontech's **Yeast Media Pouches**. The particular minimal medium that is chosen will determine which plasmids and/or activated reporters are selected for.
- **SD/–Leu** dropout supplement is used to select for the prey library plasmids. SD/–Leu dropout is so called because the medium includes every essential amino acid except for leucine, which is omitted from the formulation (or "Dropped Out"). Cells harboring Matchmaker prey plasmids are able to grow because pGADT7-Rec encodes the *LEU2* leucine biosynthesis gene, which is otherwise absent from the cell.

IV. List of Abbreviations

AD fusion library [or AD library]	A cDNA library (such as a Mate & Plate Library) constructed in an activation domain (AD) vector such that the proteins encoded by the inserts are fused to the 3' end of the Gal4 AD
AD/library plasmid	Plasmid encoding a fusion of the Gal4 activation domain and a library cDNA
AD/library protein	A protein fusion comprised of the Gal4 activation domain and a polypeptide encoded by a library cDNA
AD vector	Plasmid encoding the yeast Gal4 activation domain

Yeast Phenotypes

Ade–, or His–, or Leu–, or Trp–	Requires adenine (Ade), or histidine (His) or leucine (Leu), or tryptophan (Trp) in the medium to grow; i.e., is auxotrophic for one (or more) of these specific nutrients
LacZ+	Expresses the *LacZ* reporter gene; i.e., is positive for ß-galactosidase (ß-gal) activity.
Mel1+	Expresses the *MEL1* reporter gene; i.e., is positive for α-galactosidase (α-gal) activity

Miscellaneous

SD	Minimal, synthetically defined medium for yeast; is comprised of a nitrogen base, a carbon source (glucose unless stated otherwise), and a DO supplement
DO	Dropout (supplement or solution); a mixture of specific amino acids and nucleosides used to supplement SD base to make SD medium; DO solutions are missing one or more of the nutrients required by untransformed yeast to grow on SD medium. For example, SD/–Leu lacks the amino acid leucine.
YPDA	YPD medium supplemented with adenine hemisulfate (1X concentration = 120 µg/ml)

V. Host Strain Information

The phenotype and complete genotype of Y187 Yeast Strain (the library strain) are shown in Tables I and II. For additional information on the growth and maintenance of yeast, see the supporting Matchmaker protocols at **www.clontech.com**. We also recommend the Guide to Yeast Genetics and Molecular Biology (Guthrie & Fink, 1991).

Table I: Yeast Host Strain Genotype

Strain	Genotype[a]	Reporters	Transformation Markers	Reference
Y187[b]	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met–, URA3 : : GAL1$_{UAS}$–Gal1$_{TATA}$–LacZ MEL1*	*MEL1, LacZ*	*trp1, leu2*	Harper *et al.*, 1993

[a] The *GAL1, GAL2*, and *MEL1* upstream activating sequences (UASs) are recognized and bound by the Gal4 BD. The *trp1, his3, gal4*, and *gal80* mutations are all deletions; *leu2-3, 112* is a double mutation.

[b] The *LacZ* reporter construct was integrated into the yeast genome by homologous recombination at the *ura3-52* mutation. Recombinants were selected on SD/–Ura.

Table II: Phenotype Testing on Various SD Media

Strain	SD/–Ade	SD/–His	SD/–Leu	SD/–Trp	SD/–Ura
Y187	–	–	–	–	+
Y187 [pGADT7-T]	–	–	+	–	+

VI. Control Experiments

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Use this procedure to perform a control homologous recombination-mediated cloning in yeast BEFORE constructing and screening a two-hybrid library.

A. General Considerations

The following protocol is designed to familiarize yourself with the procedures and expected results of homologous recombination mediated cloning in Yeast. Additionally it provides you with a positive control strain that you will need for subsequent yeast two-hybrid screening.

Protocol
5–7
days

B. Protocol: Control for Homologous Recombination-Mediated Cloning Protocol

1. Materials:
   - Yeastmaker Yeast Transformation System 2 (supplied with this system, or sold separately as Cat. No. 630439)
   - Y187 Yeast Strain
   - pGADT7-Rec Cloning Vector (SmaI-linearized; 500 ng/μl)
   - SV40 Large-T PCR fragment (25 ng/μl)
   - SD/–Leu with Agar
2. Prepare
   - competent Y187 yeast cells using the Yeastmaker Yeast Transformation System 2 according to the protocol in the accompanying user manual.
   - SD/-Leu agar plates (5 x 100 mm plates; see Appendix C).
3. Combine the following DNA samples in two sterile microcentrifuge tubes and use to cotransform competent Y187 cells:
   - 1 μl pGADT7-Rec (SmaI Linearized)
   - 1 μl pGADT7-Rec (SmaI Linearized) + 3 μl SV40 large T PCR Fragment (25 ng/μl)
4. Plate 100 μl of a 1/10 dilution and 100 μl of a 1/100 dilution on SD/-Leu agar plates.
5. Incubate at 30°C for 3–5 days

   Expected results: You should see >10X more colonies when cotransforming pGADT7-Rec together with the SV40 Large-T PCR fragment compared to the vector alone. This implies that >90% of the colonies contain a pGADT7-T control plasmid created by homologous recombination.

VII. Generating the cDNA for Your Library

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Detailed instructions are provided for first-strand cDNA synthesis (Section A), cDNA amplification using long distance PCR (LD-PCR) (Section B), and column purification of ds cDNA using a CHROMA SPIN TE-400 column (Section C).

Use the following protocol for generating cDNA using Clontech's simple and highly efficient SMART™ technology (Figure 2). For a detailed description of SMART technology, refer to Appendix D.

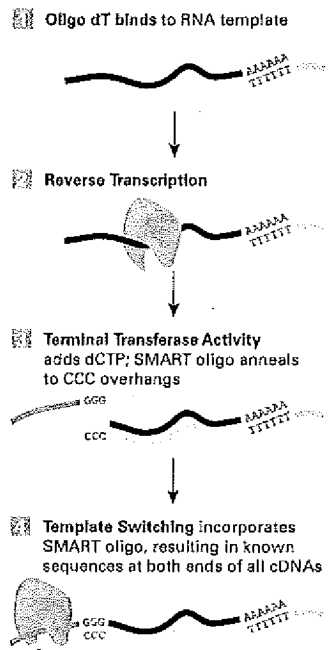

Figure 2. Mate & Plate Library construction overview. SMART cDNA synthesis generates cDNA ends with homology to pGADT7-Rec.

Protocol
1 day

A. Protocol: First Strand cDNA Synthesis

It is strongly recommended that you perform a positive control cDNA synthesis reaction with Mouse Liver Poly A+ RNA. This control verifies that all components are working properly and lets you compare to the yield and size range of the ds cDNA synthesized from your experimental RNA sample.

IMPORTANT: Do not increase the size (volume) of any of the reactions. All components have been optimized for the volumes specified.

The procedure consists of three steps:

- First-strand cDNA synthesis
- Amplification of cDNA by long distance PCR (LD-PCR)
- Column purification of ds cDNA with a CHROMA SPIN TE-400 column

In the protocol that follows, you have the option of priming first-strand cDNA synthesis with an oligo-dT (CDSIII) or random primer (CDSIII/6). The reaction conditions vary slightly (at Step 6), depending on the primer used.

VII. Generating the cDNA for Your Library continued

1. Prepare: high-quality Poly A or total RNA

Note: We recommend NucleoSpin® RNAII kits (Cat. Nos. 740955.20, 740955.50 & 740955.250) for purification of total RNA form a variety of sources, and NucleoTrap mRNA Kits (Cat. Nos. 740655 & 740656) for poly A+ mRNA enrichment from total RNA.

2. Combine and mix the following reagents in a sterile microcentrifuge tube:

Recipe

1–2 µl RNA sample (0.025–1.0 µg poly A+ or 0.10–2.0 µg total RNA)

1.0 µl CDS III or CDSIII/6 Primer

1–2 µl Deionized H_2O (to bring volume up to 4.0 µl).

4.0 µl total volume

NOTE: For the control reaction, use 1 µl (1 µg) of the control RNA.

CDSIII = Oligo-dT Primer

CDSIII/6 = Random Primer

3. Incubate at 72°C for 2 min
4. Cool on ice for 2 min only, then spin down briefly at 14,000 g for 10 sec.
5. Prepare a reaction mix containing the following components, then add this mix to the denatured, primed RNA sample from Step 4, and mix by tapping:

Recipe

2.0 µl 5X First-Strand Buffer

1.0 µl DTT (100 mM)

1.0 µl dNTP Mix (10 mM)

1.0 µl SMART MMLV Reverse Transcriptase

9.0 µl total volume

6. **ONLY if using Random Primer (CDSIII/6)** [Omit this step if using Oligo-dT (CDSIII), and continue to Step 7]

   Incubate at 25°C for 10 min at room temperature.

7. Incubate at 42° for 10 min.

NOTE: Carry out the incubation in a hot lid thermal cycler. If you are using a water bath or non-hot-lid cycler, add a drop of mineral oil to prevent loss in volume due to evaporation.

8. Add 1 µl SMART III-modified oligo, mix and incubate at 42°C for 1 hr.
9. Place the tube at 75°C for 10 min to terminate first-strand synthesis.
10. Cool to room temperature, add 1 µl RNase H (2 units).
11. Incubate at 37° for 20 min.
12. Proceed to LD-PCR amplification (Section VII.B).

NOTE: Any first -strand synthesis reaction that is not used immediately can be stored at –20°C for up to 3 months.

VII. Generating the cDNA for Your Library continued

Protocol
5 hr

B. Protocol: Amplify cDNA Using Long Distance PCR (LD-PCR)

Table III shows the optimal number of thermal cycles to use based on the amount of RNA used in the first-strand synthesis. Use the minimum number of cycles required to generate 3–6 µg of dsDNA. The optimal cycling parameters in Table III were determined using the Control Poly A+ Mouse Liver RNA; these parameters may vary with different templates and thermal cyclers. **This procedure has been tested and optimized only for Advantage 2 Polymerase Mix (Cat. No. 639201).**

Table III: Relationship between Amount of RNA and Optimal Number of Thermal Cycles

Total RNA (µg)	Poly A+ RNA (µg)	Number of Cycles
1.0–2.0	0.5–1.0	15–20
0.5–1.0	0.25–0.5	20–22
0.25–0.5	0.125–0.25	22–24
0.05–0.25	0.025–0.125	24–26

1. <u>Prepare</u>:
    - First-strand cDNA (Section VII.A)
    - Preheat thermal cycler
2. Set up **TWO** 100 µl PCR reactions for each experimental sample and one reaction for the placental control RNA:

    2 µl First-Strand cDNA (Section VII.A)
    70 µl Deionized H_2O
    10 µl 10X Advantage® 2 PCR Buffer
    2 µl 50X dNTP Mix
    2 µl 5' PCR Primer
    2 µl 3' PCR Primer
    10 µl 10X Melting Solution
    2 µl 50X Advantage 2 Polymerase Mix
    100 µl total volume

3. Begin thermal cycling using the following parameters:
    - 95°C 30 sec
    - x Cycles[a]
        95°C 10 sec
        68°C 6 min[b]
    - 68°C 5min

a Refer to Table III to estimate the number of cycles.
b Program the cycler to increase the extension time by 5 sec with each successive cycle. For example, in the second cycle, the extension should last 6 min and 5 sec; in the third, 6 min and 10 sec, and so on.

VII. Generating the cDNA for Your Library continued

4. Analyze a 7 µl aliquot of the PCR product from each sample alongside 0.25 µg of a 1 kb ladder DNA molecular weight marker on a 1.2% agarose/EtBr gel.

   Typical results obtained with Mouse Liver Poly A+ RNA after column chromatography are shown in Figure 3.

   Note: If your PCR product does not appear as expected, refer to the Troubleshooting Guide (Section X).

5. Proceed with Column Chromatography (Section VII.C) or store ds cDNA at –20°C until use.

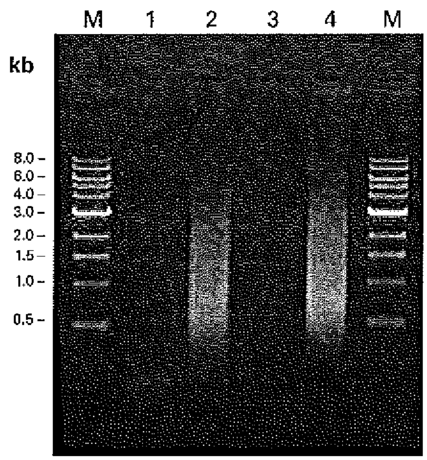

Figure 3. High-quality cDNA generated using SMART cDNA synthesis. Oligo dT-primed cDNAs were generated using the Make Your Own "Mate & Plate" Library System. cDNA synthesis was carried out with or without 1 µg of mouse liver polyA RNA (positive and negative controls, respectively). LD PCR was performed using the Advantage® 2 Polymerase Mix (with duplicate samples) and one set of products was purified (size-selected) using CHROMA SPIN+TE-400 columns. Analysis of each respective sample on a 1% agarose gel revealed that the resulting cDNAs ranged from 300 bp to 6 kb. Lanes M: 1 kb ladder molecular weight standard. Lane 1: unpurified negative control. Lane 2: unpurified positive control. Lane 3: purified negative control. Lane 4: purified positive control. Lane 4 shows reduced abundance of cDNA below 400 bp compared to Lane 2, after size selection with CHROMA SPIN+TE-400 columns.

Protocol
2 hr

C. Protocol: Purify ds cDNA with CHROMA SPIN™+TE-400 Columns

In the following protocol, a CHROMA SPIN TE-400 Column is used to select for DNA molecules >200 bp.

CHROMA SPIN Columns are packed with resin that fractionates molecules based on size. Molecules larger than the pore size are excluded from the resin. These molecules quickly move through the gel bed when the column is centrifuged, while molecules smaller than the pore size are retained and trapped inside the gel matrix. For more information about CHROMA SPIN Columns, please refer to the CHROMA SPIN Columns User Manual (PT1300-1), available at our web site at **www.clontech.com**.

1. **Prepare:**
   - ds cDNA by LD-PCR (Section VII.B; if you do not have at least 3 µg, repeat amplification step with more cycles)
   - sodium acetate (3 M)
   - ice cold ethanol (95–100%)

VII. Generating the cDNA for Your Library continued

2. Prepare one CHROMA SPIN TE-400 column for each 93 µl cDNA sample (see Figure 4).
   - Invert each column several times to resuspend the gel matrix completely, until it is homogeneous.
   - Remove the top cap.
   - Snap off the break away from the bottom of the column.
   - Place the column in a 2 ml collection tube (supplied).

   NOTE: You will use 2 columns for each library to be constructed.

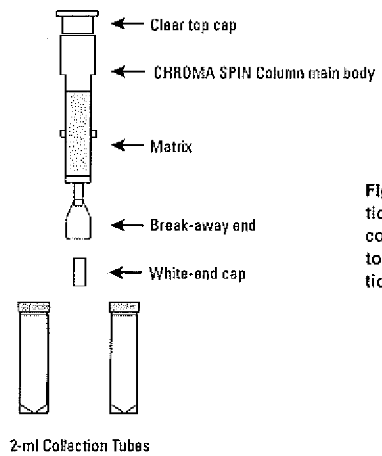

Figure 4. CHROMA SPIN column and collection tubes. Note that a conventional, tapered 1.5 ml microcentrifuge tube can be substituted for the 2 ml collection tube when collecting the purified DNA. This will allow the sample to be confined to a narrower area for easier handling. However, a 2 ml collection tube must be used when purging the equilibration buffer.

3. Centrifuge at 700 g for 5 min to purge the equilibration buffer, then discard collection tube and buffer.

   The matrix will appear semi-dry.

   NOTE: We recommend swing bucket or horizontal rotors. Fixed angel rotors can be used but there is a risk that the sample will pass down the inner side of the columns instead of through the gel matrix, resulting in inconsistent purification.

4. Replace spin column in second collection tube and apply your 93 µl sample to the CENTER of the flat surface of the gel matrix.

   NOTE: Do not allow sample to flow along inner wall of the column.

5. Centrifuge at 700 g for 5 min, your purified sample is now in the collection tube.
6. Combine your two purified samples into a single microcentrifuge tube and ethanol-precipitate the cDNA:
   - Add 1/10th vol 3 M sodium acetate.
   - Add 2.5 vol of ice-cold ethanol (95–100%).
   - Place in –20°C freezer for 1 hr.
   - Centrifuge at 14,000 rpm for 20 min at room temperature.
   - Discard the supernatant; do not disturb the pellet.
   - Centrifuge briefly at 14,000 rpm and remove remaining supernatant.
   - Air dry the pellet for 10 min.
7. Resuspend the cDNA in 20 µl deionized water.

   The cDNA is now ready for library construction by *in vivo* recombination in yeast (Section VIII).

   NOTE: At this point you should have 2–5 µg of ds cDNA

VIII. Construction of a Two-Hybrid Library

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING
Detailed instructions are provided for constructing a "Mate & Plate" yeast two-hybrid library (Section B).

A. General Considerations for Two-Hybrid Library Construction & Screening

There are two ways to use this kit to make and screen a Matchmaker Two-Hybrid Library.

1. **Recommended Protocol**
   - Make a Mate & Plate library in Y187 Yeast Strain (see Section VIII.B, below) and screen against the bait by yeast mating [see the Matchmaker Gold Yeast Two-Hybrid System User Manual (PT4084-1)]. You will note that the mating procedure is very easy—simply mix a concentrated bait culture with 1 ml of your Mate & Plate library constructed using the protocol and incubate overnight before plating on SD-Leu/-Trp/X-α-Gal/AbA selective media.
   - See the Matchmaker Gold Yeast Two-Hybrid System User Manual (PT4084-1) for instructions on screening a library using yeast mating at **www.clontech.com**
2. **Alternative Protocol**
   - Simultaneously make and screen a library by library-scale cotransformation of Bait and Prey. Prepare ds cDNA according to the protocol in Section VII and cotransform together with your bait directly into Y2HGold competent cells. Visit **www.clontech.com** for a support protocol.

The advantage of the recommended protocol is that it provides frozen stocks of the library to screen multiple baits. The alternative protocol will not result in a frozen library stock but is a quicker procedure. You can follow this protocol if you do not plan to screen a library multiple times.

B. Protocol: Creating a Mate & Plate Library

Protocol 5–7 days

1. Prepare:
   - competent Y187 yeast cells using the Yeastmaker Yeast Transformation System 2 according to the protocol in the accompanying user manual.
   - 20 µl ds cDNA (Section VII)— The amount of ds cDNA should be 2–5 µg.
   - The following SD Agar Plates (Appendix C)
     - SD/–Leu with Agar (100 x 150 mm plates)
     - SD/–Leu with Agar (5–10 x 100 mm plates)
   - YPDA/25% Glycerol (Freezing Medium; see Section C of Appendix C)

   NOTE: Kanamycin sulfate can be added to all yeast media at a final concentration of 50 µg/ml to stop bacterial contamination (see Appendix C, Section D).

   - sterile glass beads (5 mm)
2. Follow the library-scale transformation protocol in the Yeastmaker Yeast Transformation System 2 User Manual to cotransform the following into competent Y187 yeast cells.
   - 20 µl ds cDNA (Section VII)— The amount of ds cDNA should be 2–5 µg
   - 6 µl pGADT7-Rec (0.5 µg/µl))
3. As specified in the transformation protocol in Step 2, resuspend the transformed cells in 15 ml of 0.9% (w/v) NaCl.

VIII. Construction of a Two-Hybrid Library continued

4. Spread 100 µl of 1/10 and 1/100 dilutions on SD/–Leu 100 mm agar plates, incubate at 30°C for 3–4 days, and determine the **number of independent clones** in your library. The number of independent clones is an indication of library complexity.

   Refer to Section X to improve your transformation efficiency.

Number of Independent Clones = No. of cfu/ml on SD/–Leu x resuspension volume (15ml)

- This value will determine the transformation efficiency.
- If you have made a library of 2 million independent clones, you will have 133 colonies on the 1/100 dilution plate.
- Only proceed to Step 6 if you have determined that you have >1 million independent clones
- If your library does not have >1 million independent clones, refer to the Section X to learn how to improve transformation efficiency and the quality of your cDNA.

5. Spread the remainder on 150 mm SD/–Leu Plates
   - 150 µl per plate on ~100 plates
   - Incubate at 30°C for 3–4 days)
6. Harvest and Pool Transformants from Step 5.
   a. Chill plates at 4°C for 3–4 hr.
   b. Add 5 ml of Freezing Medium
   c. Use sterile glass beads to detach the colonies from the plate.
   d. Combine all liquids in a single sterile flask (you should have a combined total of 0.5 L)
   e. Using a hemocytometer, estimate the cell density. If the cell density is <2 x 10^7 per ml, reduce the volume of the suspension by centrifugation.
   f. Aliquot your library into several 1 ml aliquots for short-term use and a few 50 ml aliquots for long-term storage. Store at –80°C.
7. Use a single 1ml aliquot for library screening using the Matchmaker Gold Yeast Two-Hybrid System User Manual (PT4084-1).

NOTE: When thawing libraries from storage, always recalculate the titer to confirm that the titer remains >1x107 cfu/ml

IX. References

- For general reviews on yeast two-hybrid systems, see Allen *et al.*, 1995; Bartel *et al.*, 1993a, 1993b; Bartel & Fields, (1997); Fields, 1993; Fields & Sternglanz, 1994; Fritz & Green, 1992; Guarente, 1993; Hopkin, 1996; Luban & Goff, 1995; McNabb & Guarente, 1996; Mendelsohn & Brent, 1994.
- An extensive list of Matchmaker System citations can be obtained from our website (**www.clontech.com**).
- For additional two-hybrid references, see the Golemis lab Web Site (http://www.fccc.edu:80/research/labs/golemis) or use MedLine (http://www.ncbi.nlm.nih.gov/PubMed/medline.html) and search under key words "two-hybrid."

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X. Troubleshooting Guide

PROBLEM	CAUSE	SOLUTION
Low yield of ds cDNA	One or more essential reagents may have been inadvertently omitted from the first-strand or ds cDNA synthesis steps.	Repeat both steps, being careful to check off every item as you add it to the reaction.
	Low yields of ds cDNA may be due to PCR undercycling.	• If you suspect undercycling, incubate the PCR reaction mixture for two more cycles and recheck the product. • If you already used the maximum recommended number of cycles, increase by three more cycles. • If additional cycles do not improve the yield of PCR product, repeat the PCR using a fresh aliquot of the first-strand product.
	A low yield of ds cDNA may also be due to a low yield of first-strand cDNA. Possible problems with the first-strand reaction include: • a mistake in the procedure (such as using a suboptimal incubation temperature or omitting a component), or insufficient RNA in the reaction. • It is also possible that the RNA has been partially degraded (by contaminating RNases) before the first-strand synthesis	• Problems with the first-strand cDNA synthesis can be more easily diagnosed if you perform parallel reactions using the control RNA provided in the kit. • If good results were obtained with the control RNA but not with your experimental RNA, then there may be a problem with your RNA.
Size distribution of ds cDNA is less than expected	your RNA starting material may be degraded, very impure, or too dilute.	• Check the quality and quantity of your RNA by running a sample on a gel. If the RNA seems too dilute, but otherwise of good quality, restart the experiment using more RNA. • If the RNA seems degraded, restart the experiment using a fresh lot or preparation of RNA. • Also, check the stability of your RNA by incubating a small sample at 37°C for 2 hr. Run it on a gel, parallel to a fresh (unincubated) sample. If the RNA appears to be unstable, it will yield poor results. If this is the case, re-isolate the RNA using a different method (see Related Products). • Problems with your RNA are easily diagnosed if you perform parallel reactions using the control RNA provided in this kit.

X. Troubleshooting Guide continued

PROBLEM	CAUSE	SOLUTION
Presence of low molecular weight (<0.1 kb) material in the ds cDNA product	• The raw cDNA (e.g., before size fractionation) is expected to contain some low-molecular-weight DNA contaminants, including unincorporated primers, SMART oligonucleotides, and very short PCR products. • However, these small fragments are generally removed from the ds cDNA preparation in the size fractionation step using the columns provided. • Note that a preponderance of low-molecular-weight (<0.1 kb) material in the raw PCR product may be indicative of overcycling.	If you suspect overcycling, then the PCR step must be repeated with a fresh sample of first-strand cDNA, using 2–3 fewer cycles.
Presence of low molecular weight (<0.1 kb) in the size-fractionated ds cDNA	CHROMA SPIN columns are designed to remove low-molecular-weight cDNA fragments, small DNA contaminants, and unincorporated nucleotides from the cDNA. The resolving function of the column will be diminished if the gel matrix becomes dry. In drying, the matrix body may shrink away from the inner wall of the column casing. The ds cDNA mixture can then flow down the sides of the column, allowing small contaminants to elute with the cDNA.	The column should be stored and used at room temperature. If it is chilled at 4°C and then warmed to room temperature for use, bubbles may form, which interfere with the proper functioning of the column. Extreme, uneven deposition of the ds cDNA mixture on the surface of the column can cause inefficient separation of ds cDNA from low-molecular-weight contaminants.
Low transformation efficiency	DNA is not sufficiently pure	Check the purity of the DNA and, if necessary, repurify by ethanol precipitation.
	The fusion protein encoded by the DNA-BD/bait plasmid (Two-Hybrid System) may be toxic.	Try using a vector that expresses lower levels of the fusion, such pGBT9.
	Improper media preparation	Remake media and test with control transformations. Check the efficiency using the pGBT9 Control Plasmid. Plate on SD/–Trp. The transformation should yield ≥1 x 10^5 colonies/µg DNA.
	Slow-growing Y187 yeast cells	Streak to single colonies, innoculate 4 large colonies into YPDA broth, and select the fastest growing to make competent cells.
Yeast two-hybrid screening problems		See Matchmaker Gold Yeast Two-Hybrid System User Manual (PT4084-1) at **www.clontech.com**

Appendix A: Library Titering

A. General Considerations:

Attention

- Diluted libraries are always less stable than undiluted libraries. Therefore, once the library dilutions are made, plate them within the next hour, before misleading reductions in titer can occur.
- Use proper sterile technique when aliquoting and handling libraries.
- Design and use appropriate controls to test for cross-contamination.
- Always use the recommended concentration of antibiotic (i.e., kanamycin) in the medium to suppress growth of contaminating bacteria.

Protocol 4–6 days

B. Library Titering

1. Materials
   - YPDA Broth (Appendix C)
   - SD/–Leu (100 mm plates) (Appendix C)
   - Sterile glass spreading rod, bent Pasteur pipette, or 5 mm glass beads for spreading culture on plates.
2. Transfer the 10 µl library aliquot (reserved from Section VIII) to 1 ml of YPDA Broth in a 1.5 ml microcentrifuge tube. Mix by gentle vortexing. This is **Dilution A** (dilution factor = 10^{-2}).

NOTE: Matchmaker Gold Libraries are extremely viscous, so pay special attention during aliquoting.

3. Remove 10 µl from Dilution A, and add it to 1 ml of YPDA Broth in a 1.5 ml microcentrifuge tube. Mix by gentle vortexing. This is **Dilution B** (dilution factor = 10^{-4}).
4. Add 10 µl from Dilution A to 50 µl of YPDA Broth in a 1.5 ml microcentrifuge tube. Mix by gentle vortexing. Spread the entire mixture onto an SD/–Leu plate.
5. Remove 50 µl aliquots from Dilution B and spread onto separate SD/–Leu plates as above.
6. Invert the plates and incubate at 30°C for 3–5 days.

   NOTE: Colony size will vary, depending on the insert.
7. Count the number of colonies on plates having 30–300 colonies.
8. Calculate the titer (cfu/ml) as follows:

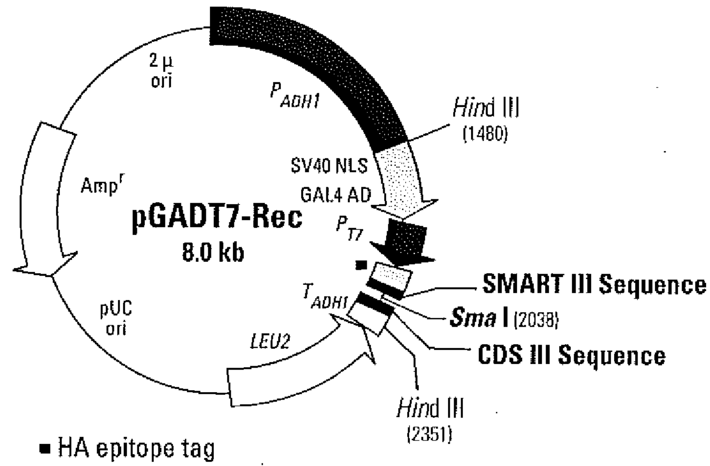
$$\frac{\text{Number of colonies}}{\text{plating volume (ml)} \times \text{dilution factor}} = \text{cfu/ml}$$

NOTE: Due to slight variability in pipettes and pipetting techniques, a 2–5-fold range in titer calculations is not unusual.

- Example calculation:
- No. of colonies on plate = 100
- Plating volume = 0.05 ml
- Dilution factor = 10^{-4}

$$\frac{100}{0.05\text{ ml} \times 10^{-4}} = 2 \times 10^{7}\text{ cfu/ml}$$

Appendix B: Plasmid Information



SMART III™ terminus

1858 — MATCHMAKER 5' AD LD-Insert Screening Amplimer → ; GAL4 Activation Domain, a.a. 881 ; T7 Sequencing Primer ; T7 Promoter

CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CCA AAA AAA GAG ATC TTT AAT ACG ACT

1915 — START *in vitro* ; HA Epitope Tag

CAC TAT AGG GCG AGC GCC GCC ATG GAG TAC CCA TAC GAC GTA CCA GAT TAC GCT

1969 — SMART III Primer

CAT ATG GCC ATG GAG GCC AGT GAA TTC CAC CCA AGC AGT GGT ATC AAC GCA GAG TGG

2026

CCA TTA TGG CCC

CDS III terminus

2038 — CDS III Primer

GGG AAA AAA CAT GTC GGC CGC CTC GGC CTC TAG AGG GTG GGC ATC GAT ACG GGA TCC

2095 — STOP

ATC GAG CTC GAG CTG CAG ATG AAT CGT AGA TAC TGA AAAACCCCGCAAGTTCACTTC

← MATCHMAKER 3' AD LD-Insert Screening Amplimer

2152

AACTGTGCATCGTGCACCATCT

← 3' AD Sequencing Primer

Figure 5. Restriction Map and Cloning Sites of pGADT7-Rec. A unique restriction site (Sma I) is shown in bold. The Make Your Own "Mate & Plate" Library System (Cat. No. 630490) contains the Sma I-linearized form of this vector, the form used for recombination-mediated cloning in yeast.

Appendix C: Yeast Growth Media & Supplements

A. Ready-to-go Media Pouches Available from Clontech

Clontech offers media sets with a complete assortment of mixes in convenient, "ready-mixed" foil pouches, for use with the Matchmaker Gold Yeast Two-Hybrid System. See Table IV for a list of the components of the **Yeast Media Set 2** (Cat. No. 630494) and the **Yeast Media Set 2 Plus** (Cat. No. 630495). The Yeast Media Set 2 Plus also contains the additional media supplements **Aureobasidin A** and **X-α-Gal**, which are required for yeast two-hybrid screening using the Matchmaker Gold Yeast Two-Hybrid System, but not with this user manual.

- See Table V for information for purchasing each of the media mixes separately, in packs of 10 pouches.
- See Table VI for information on preparing all additional required media supplements and purchasing Aureobasidin A and X-α-Gal separately.

Table IV: Components of Yeast Media Set 2 & Yeast Media Set 2 Plus

Media Pouch	Quantity of Pouches Supplied	Volume of Media Each Pouch Makes
YPDA Broth	2	0.5 L
YPDA with Agar	1	0.5 L
SD/–Leu Broth	1	0.5 L
SD/–Leu with Agar	1	0.5 L
SD/–Trp Broth	1	0.5 L
SD/–Trp with Agar	1	0.5 L
SD/–Leu/–Trp with Agar	10	0.5 L
SD/–Ade/–His/–Leu/–Trp with Agar	1	0.5 L
Additional Components in Yeast Media Set 2 Plus		
X-alpha-Gal	250 mg	—
Aureobasidin A	1 mg	—

Table V: Individual Yeast Media Pouches for Matchmaker Gold Protocols

Yeast Media Pouches	Clontech Cat. No.	Quantity of Pouches Supplied	Volume of Media Each Pouch Makes
Rich Media (for Routine Culturing of Untransformed Yeast)			
YPDA Broth	630306	10	0.5 L
YPDA with Agar	630307	10	0.5 L
Minimal Media Single Dropouts (SDO)			
SD–Trp Broth	630308	10	0.5 L
SD–Trp with Agar	630309	10	0.5 L
SD–Leu Broth	630310	10	0.5 L
SD–Leu with Agar	630311	10	0.5 L
Minimal Media Double Dropouts (DDO)			
SD–Leu/–Trp Broth	630316	10	0.5 L
SD–Leu/–Trp with Agar	630317	10	0.5 L
Minimal Media Triple Dropouts (TDO)			
SD–His/–Leu/–Trp Broth	630318	10	0.5 L
SD–His/–Leu/–Trp with Agar	630319	10	0.5 L
Minimal Media Quadruple Dropouts (QDO)			
SD–Ade/–His/–Leu/–Trp Broth	630322	10	0.5 L
SD–Ade/–His/–Leu/–Trp with Agar	630323	10	0.5 L